RAP1-like binding activity in islet cells corresponds to members of the Sp1 family of transcription factors

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Abstract Deletion and mutational analyses of the gastrin promoter have identified a binding site for the yeast transcription factor RAP1 relevant for transcriptional activation in islet cells. We here report that the mammalian transcription factors binding to this site in islet cells are the Sp transcription factor members Sp1 and Sp3. Furthermore, functional analyses revealed Sp1- and Sp3-mediated transcriptional activation of gastrin. These data reveal that the zinc finger proteins Sp1 and Sp3 do have similar binding specificities as the multifunctional yeast RAP1 protein.

Key words: Gastrin promoter; Islet cell; RAP1; Sp-related transcription factor

1. Introduction

RAP1 is a highly abundant multifunctional DNA-binding protein which effects both transcriptional activation and silencing in yeast dependent on the context of the promoter. RAP1 also binds to autonomously replicating sequences and telomeres of yeast chromosomes to regulate cell division [1–3]. Gene regulation is highly conserved between yeast and mammalian cells. We recently reported that the rat gastrin promoter contains a yeast RAP1-binding site within the *cis*-regulatory domain GRD (gastrin regulatory domain) controlling gastrin gene transcription in islet cells [4]. Point mutations in the rat gastrin RAP1-binding site abolished RAP1 binding and decreased transcriptional activation. Recent data suggested that the CACC box in the human gastrin promoter, corresponding to the RAP1 site in the rat gastrin promoter, is also critical for gastrin activation in islet cells [5]. Furthermore, DNA–protein-binding analyses revealed a DNA-binding activity with RAP1-like binding specificity in islet cells, suggesting a mammalian homologue RAP1-like protein [4]. Gastrin presents an oncofetal expression pattern in islet cells. The underlying molecular mechanisms of gastrin reexpression in transformed islet cells are, however, still unknown. Therefore, transcriptional activators binding to the islet regulatory domain (GRD) could be pathogenetically relevant. This paper reports that the RAP1-like binding activity in islet cells is zinc-dependent and corresponds to the mammalian zinc finger proteins Sp1 and Sp3, members of a multigene family of GC box binding proteins with similar structural features [6]. Furthermore, cotransfection experiments revealed Sp1- and Sp3-mediated transcriptional activation, dependent on the RAP1-binding site. These data suggest that Sp1 and Sp3 are the two mammalian proteins acting through the RAP1 site of the gastrin promoter.

2. Materials and methods

2.1. Gastrin reporter gene construction

The 1300 gastrin reporter gene construct hugas1300 was created by subcloning the *Eco*RI–*Pst*I fragment of the human gastrin gene (1300 bp of 5′-flanking DNA and the first exon) into the promoterless luciferase vector pGL2-basic (Promega). Deletion constructs hugas119WT and hugas119mt were created using the polymerase chain reaction (PCR) and the hugas1300 construct as template. The hugas 119mt construct contains a single point mutation in the RAP1-binding site as described [4]. Cohesive ends were generated on the PCR products by restriction digest before ligation into the pGL2-basic vector. Correct insertion was verified by dideoxy sequencing.

2.2. Cell culture and DNA transfection

Rat insulinoma RIN38A and RINB6 cells were cultured as described [7,8]. Schneider cells *SL2* [9] were maintained in Schneider medium supplemented with 10% FCS at 25°C. One day prior to transfection, cells were plated in a mixture of Schneider/DE22 medium (1:3, supplemented with 10% FCS) onto 6 cm plastic dishes at a density of 4×10^6 cells/plate and transfected by the calcium phosphate method described [10]. Each plate received 8 μg of hugas reporter construct, 50 ng of either expression plasmid pPac, pPacSp1, pPacSp3 or pPacSp4 [11] and 4 μg of β-galactosidase expression plasmid p97b as internal reference [11]. Variable amounts of expression plasmids were compensated for with the plasmid pPac. Twenty-four hours after addition of DNA the medium was replaced by Schneider medium and 24 h later cells harvested. Luciferase activity was normalized to β-galactosidase activity for plate-to-plate variations in transfection efficiency.

2.3. Bacterial and nuclear extracts

Sp3 cDNA expression plasmids pET-3c/Sp3r (cDNA sense orientation) or pET-3c/Sp3f (cDNA inverted oritentation) were transformed into *E. coli* BL21 (LysS), grown and expression induced with IPTG as described [12]. Bacterial extracts containing recombinant protein were prepared according to Kadonaga et al. [13]. Recombinant Sp1 was purchased (Promega). Nuclear extracts from cell lines were prepared by a modified method of Dignam [4]. Protein concentration of the extract was measured by the Bradford procedure [14].

2.4. Electrophoretic mobility shift assays (EMSA)

Single-stranded oligonucleotides were labeled with [^{32}P]ATP using T4 DNA kinase, hybridized with the complementary strand and purified by NucTrap Push Column (Stratagene). Sense oligonucleotides used in this study were (hCACC) -114 GTGACCCCACCCCATT′-97, rGRDWT -110 CCACACCCATTTCTCTCGCTGTGGGAGT-CTG -78, RAP1 -112 CTCCACACCCATTTCTC -86. Binding reactions using nuclear cell extracts were performed as described [4]. Polyclonal rabbit antisera against Sp1 and Sp3 proteins were generated using bacterially expressed Sp1 and Sp3 proteins as described [6,11]. Antibodies were added to the binding assay mixture 30 min before addition of radiolabeled probe.

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2.5. DNase I protection experiments

A DNA fragment spanning nucleotides −201 to +35 of the human gastrin promoter was generated by PCR using endlabeled sense oligonucleotide 5′-AACTCCCCTATCCTTCCC-3′, unlabelled antisense oligonucleotide 5′-CTGCAGAGCTGGGAGGTG-3′ and the hugas1300 construct as a template. The probe (30 000 cpm) was mixed with 50 ng of purified Sp1 (Promega) or 10 μg of bacterial extracts in a buffer as described [11]. DNase I (0.1 U) was added, incubated for 60 s at 20°C prior to terminating the reaction. After phenol extraction and ethanol precipitation, DNA fragments were resolved on a 6% denaturing polyacrylamide gel. G and G+A sequencing reactions were performed as described [15].

3. Results

3.1. Binding of Sp1-related proteins to the RAP1 site in the rat gastrin promoter

An EMSA using the nuclear islet cell extracts and the rat GRD (rGRDWT) sequence containing the RAP1-binding site as probe revealed that RAP1-like binding activity, corresponding to complex C1 [4] was zinc-dependent (Fig. 1A). Although the RAP1 site does not contain the classic Sp1 consensus sequence, Sp1 is capable of recognizing many GC-rich divergent sequences including GT boxes and CACC motifs [16–18]. To investigate whether Sp1 was a component of RAP1-binding activity, we performed EMSAs using islet nuclear extracts and the RAP1 recognition site sequence as the probe (RAP1) in the presence or absence of antibody raised against bacterially expressed Sp1 [11] (Fig. 1B). Competition experiments revealed that complex C1 formation is dependent on an intact RAP1 binding site (data not shown). Addition of Sp1 antiserum resulted in a lower migrating supershifted complex (*SS*) and revealed complex C1 as a composition of at least two retarded bands, the major band corresponding to Sp1 (Fig. 1B, lanes 2 and 3). Addition of antiserum against Sp3, with identical binding characteristics as Sp1, resulted in the shift of a weak migrating complex (Fig. 1B, lanes 4 and 5). Combined addition of Sp1 and Sp3 antisera resulted in a high molecular weight supershifted complex that could not migrate into the gel (Fig. 1B, lane 6), while the

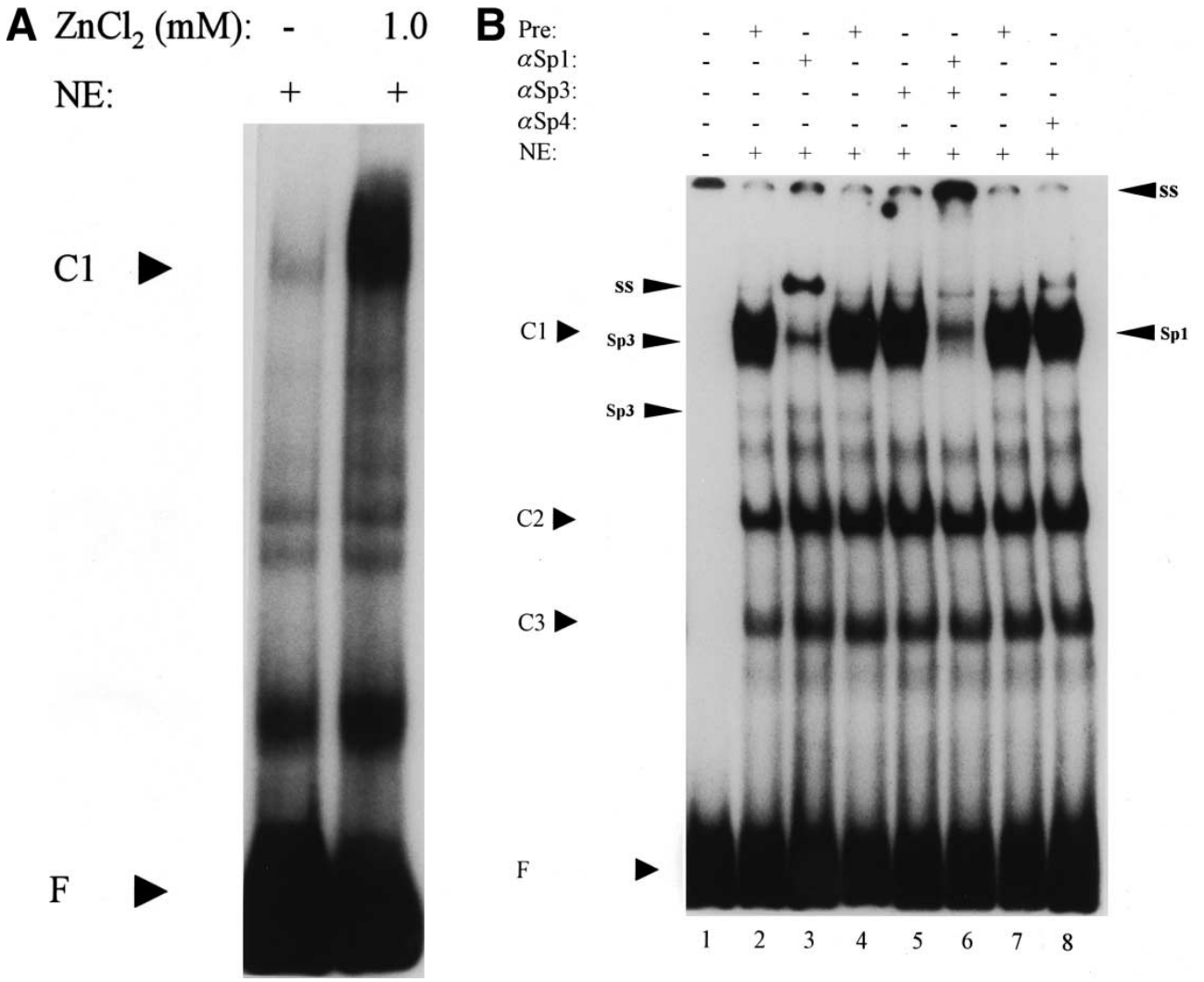

Fig. 1. Sp1 and Sp3 bind to the RAP1 element in the rat gastrin promoter. A: EMSA was performed using nuclear extracts of RIN B6 cells and the end-labelled rGRD sequence as probe. Complex formation C1 corresponding to RAP1-like binding activity in islet cells is dependent on the presence of 1.0 mM or absence (−) of $ZnCl_2$, suggesting binding of zinc finger protein (s). The other complexes formed are RAP1 independent [4]. B: Identification of Sp1 and Sp3 as components of RAP1-like binding activity. EMSAs were performed using RIN38A nuclear extracts and the RAP1-binding site as the labelled probe. Antisera against Sp1 (αSp1; lanes 3 and 6), against Sp3 (αSp3; lanes 5 and 6), against Sp4 (αSp4; lane 8), and appropriate pre-immune sera (pre; lanes 2, 4 and 7) were included into the binding reactions as indicated above each lane. Supershifted complexes are indicated SS. C, DNA–protein complex formation; F, free migrating probe.

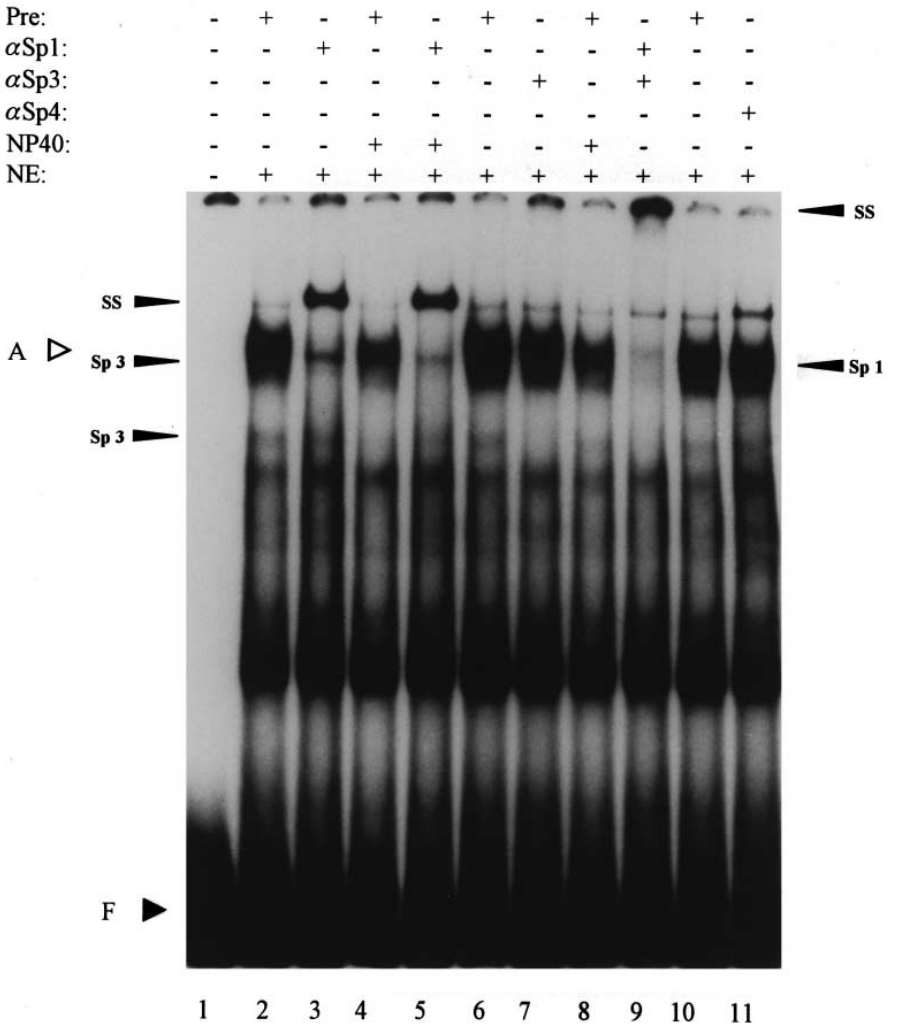

Fig. 2. Sp1 and Sp3 are components binding to the CACC element in the human gastrin promoter. EMSA were performed using RIN38A nuclear extracts and the human CACC/RAP1 site as labelled probe. Antisera against Sp1 (αSp1; lanes 3, 5 and 9), against Sp3 (αSp3; lanes 7 and 9), against Sp4 (αSp4; lane 11), and appropriate pre-immune sera (pre; lanes 2, 4, 6, 8 and 10) were included in the binding reactions as indicated above each lane. Supershifted complexes are indicated SS. NE, nuclear extracts; A, DNA–protein complex; F, free migrating probe.

faster migrating Sp3 complex and the major part of complex C1 disappeared. Addition of Sp4 antiserum revealed no gross change of any complex (Fig. 1B, lanes 7 and 8) except a faint supershifted complex comigrating with supershifted Sp1. These findings suggest that the major part of RAP1 binding consists of Sp1 and to a lesser extent of Sp3. A potential third protein comigrating in complex C1 remains to be identified. The Sp3 complex might reflect binding of the 58 kDa or/and 60 kDa Sp3 polypeptides, while the 97 kDa Sp3 polypeptide is suggested to comigrate in complex C1 [11]. The islet proteins involved in the indicated faster migrating complexes C also represent binding activities dependent on an intact RAP1-binding site, although their nature remains to be determined.

3.2. *Sp1 and Sp3 as components binding to the CACC element in the human gastrin promoter*

The CACC element in the human gastrin promoter corresponds to the RAP1 site in the rat promoter and revealed RAP1-like binding specificity [4,5]. EMSA demonstrated a major specific zinc-dependent binding activity in islet cells to the CACC element (data not shown), indicated here as complex A. Binding of Sp1-related proteins was immunologically analyzed in an EMSA using islet nuclear extracts and the CACC element as the probe. Addition of Sp1 antiserum resulted in a supershifted complex *ss* (Fig. 2, lanes 2 and 3) with concomitant absence of the major part of complex A, suggesting Sp1 binding. In the presence of Sp3 antiserum, a weak complex (lanes 6 and 7) corresponding to Sp3 was shifted. Combined addition of Sp1 and Sp3 antisera resulted in a high molecular mass complex not entering the gel (lane 9), while the Sp3 complex and complex A were shifted. While addition of Sp4 antiserum revealed no gross changes in complex formation (lanes 10 and 11), a weak complexed band (*ss*, right site) became visible comigrating with shifted Sp1, that could result from a small amount of supershifted Sp4, although this has to be further investigated. Therefore, Sp1 and Sp3 also bind to the CACC element with Sp1 constituting the major binding component.

3.3. *The proximal human gastrin promoter contains three binding sites for members of the Sp transcription factor family*

To delineate binding sites for Sp proteins in the proximal human promoter, recombinant Sp1 and Sp3 proteins were

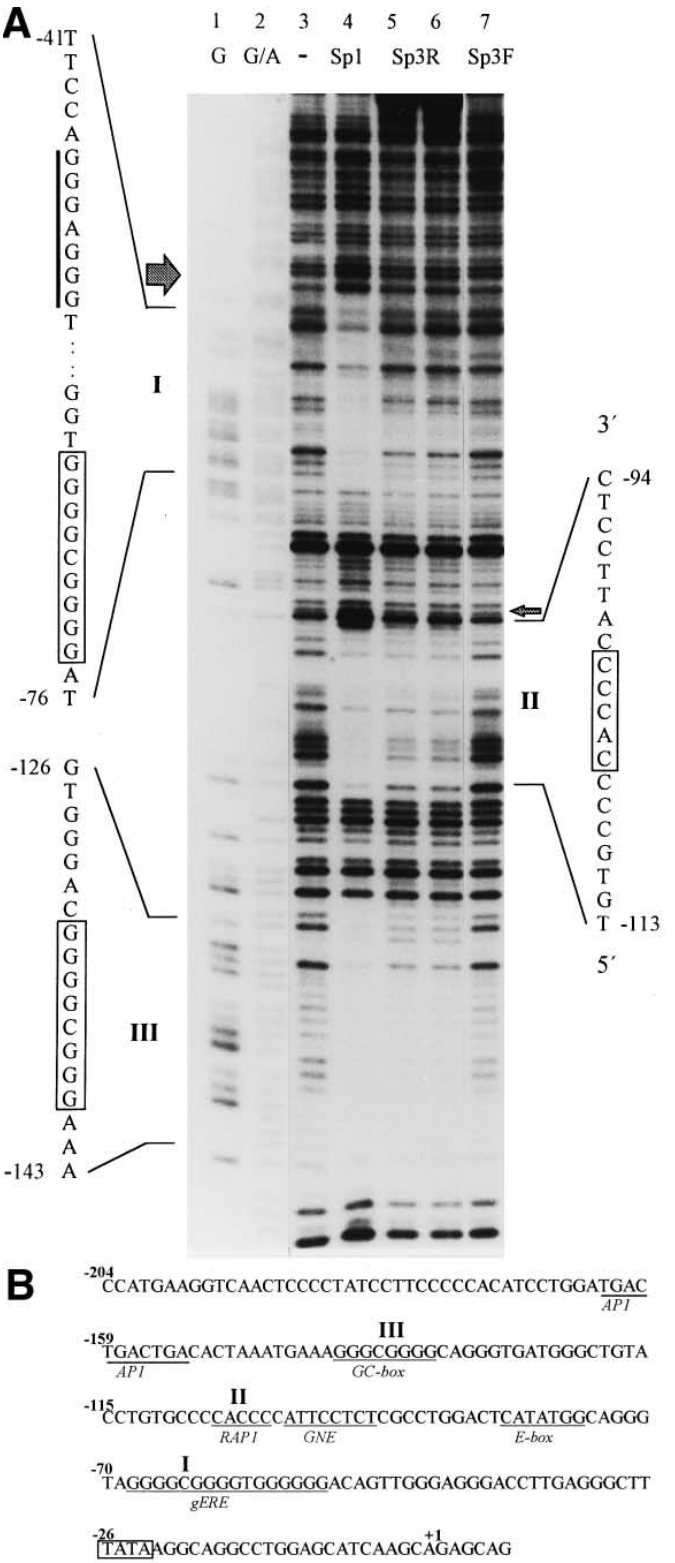

Fig. 3. DNase I protection of the CACC/RAP1-like element in the human gastrin promoter by recombinant Sp1 and Sp3. A: Reactions were performed on the coding strand of 201 bp of 5′ flanking sequence of the gastrin promoter. The labeled DNA fragments were DNase I digested in the absence (−) or presence (+) of recombinant Sp1 (Sp1), Sp3 (Sp3R) or extracts produced with a Sp3 cDNA in inverse orientation (Sp3F). Alongside the autoradiographs protected regions are boxed and indicated I−III, numbers refer to the positions relative to the transcription start site. G and G+A refer to Maxam Gilbert sequencing reactions. B: Schematic representation of Sp-binding sites in the gastrin promoter context. I–III refer to the protected sequences as demonstrated in (A).

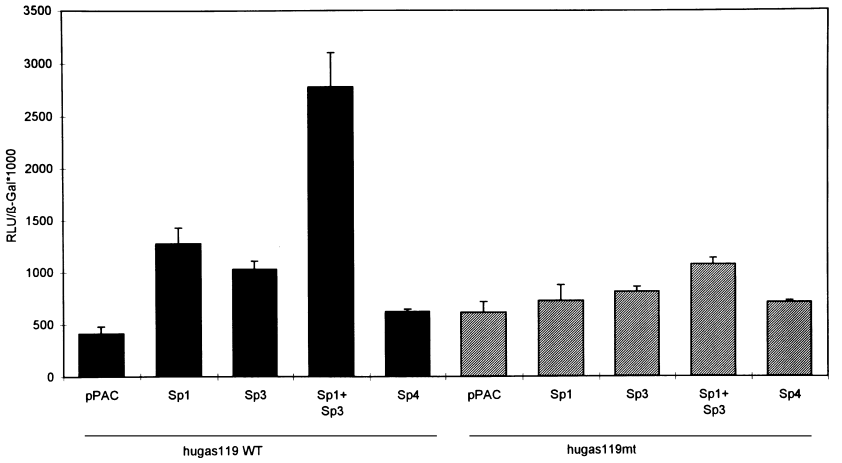

Fig. 4. Effect of Sp1 and Sp3 expression on gastrin promoter activity. SL2 cells were transiently cotransfected with 8 μg of gastrin reporter deletion construct hugas119 or the construct hugas119mt, containing a single point mutation in the RAP1-binding site [4], 50 ng of the expression plasmids pPacSp1, pPacSp3, pPacSp4 or vector pPac [11] and 4 μg of β-galactosidase expression plasmid p97b as internal reference. Combined cotransfection was performed with 50 ng of each expression plasmid. Variable amounts of expression plasmids were compensated for with the plasmid pPac. Luciferase activity was normalized to β-galactosidase activity for plate to plate variations in transfection efficiency.

tested for binding in a DNase I footprint experiment. A DNA fragment spanning nucleotides −201 to +35 was incubated with purified Sp1 or Sp3, subjected to partial DNase I digestion and analyzed (Fig. 3A). Addition of Sp1 revealed three distinct regions (I–III) of nuclease protection, each binding site covering 12–19 bp and centered around a single GC rich sequence (sites I and III) and the CACC element/RAP1-binding site (site II) as shown in Fig. 3A (lane 4). Sp3 essentially revealed an identical protection pattern except, that only a weak protection was detected at site I (Fig. 3A, lanes 5 and 6). No binding was observed with a Sp3 expression construct in inverse orientation (Fig. 3A, lane 7). The localization of the Sp-binding sites in the proximal gastrin promoter are summerized in Fig. 3B. Site I corresponds to the 5′ part of the EGF response element gERE [19]. Site II corresponds to the CACC- or RAP1-binding site, while site III was suggested to confer cAMP response synergistically with site I in GH4 cells [20]. These data confirm binding of both, Sp1 and Sp3 bind to the RAP1-like site (CACC element) in the human gastrin promoter.

3.4. *Sp1- and Sp3-mediated transcriptional activation depends on the RAP1-binding site*

Deletional analyses suggested that the RAP1/CACC element in the human gastrin promoter is a positive regulatory element [5]. Functional activity of transfected Sp1 cannot be studied unambiguously in islet cells, since they contain endogenous Sp proteins. To determine whether Sp1 and Sp3 act as transcriptional regulators binding to the RAP1 element, we performed gene transfer experiments into *Drosophila melanogaster* Schneider cells (SL2) that lack endogenous Sp factors [9]. Luciferase reporter constructs contained the intact gastrin 5′-flanking sequence spanning 119 bp including the first exon. A mutant reporter construct hugas119mt contained a single base pair mutation critical for RAP1-like binding [4,5]. Reporter constructs were transfected into SL2 cells along with one of the expression plasmids pPacSp1, pPacSp3, pPacSp4 or pPac. Expression was confirmed using a BCAT-2 construct containing two Sp1-binding sites in tandem array from the HTLV-III promoter ([10], data not shown). The hugas119 intact reporter construct comprising two binding sites for Sp1 was activated 3-fold by cotransfected Sp1, while Sp1 cotransfection only revealed a minor effect on the mutant hugas119mt reporter construct (Fig. 4). This result suggested that Sp1-mediated transcriptional activation was mainly due to the intact RAP1 element. The weak transcriptional activation of the hugas119mt reporter construct is due to the intact Sp1-binding site I of the gERE element, since further deletion of this site completely prevented Sp1-mediated activation (data not shown). Coexpression of Sp3 revealed a 2.4-fold induction of transcription using the hugas119, while only a slight increase was observed using the mutant hugas119mt reporter construct. Combined cotransfection of Sp1 and Sp3 revealed an almost 7-fold increase in transcriptional activation of the intact reporter plasmid hugas119. In contrast, only a slight increase of promoter activity was observed using the mutant reporter construct hugas119mt. Cotransfection of Sp4 revealed no transcriptional effect on either promoter construct. These data demonstrate, that both, Sp1 and Sp3 activate gastrin gene transcription, while Sp4 has no effect. Furthermore, Sp1- and Sp3-mediated transcriptional activation was dependent on an intact RAP1-binding site, supporting the DNA–protein binding analyses.

4. Discussion

Previous studies have identified a yeast RAP1-binding site within the GRD *cis*-regulatory sequence of the rat and human gastrin promoter [4]. Detection of a RAP1-like binding activity confering transcriptional activation of the gastrin promoter in islet cells suggested a mammalian transcription factor with similar binding specificity like yeast RAP1 [4,5]. Here, we report that Sp1 and Sp3 are components of the RAP1-like binding activity in islet cells. Furthermore, footprinting analysis identified three Sp1-binding sites in the proximal gastrin promoter (Fig. 3) correponding to the gERE site [19], to the RAP1 site [4,5] and a cAMP responsive element relevant in GH4cells [20]. These data are consistent with previous reports suggesting that Sp1 binding to the 5′ part of the gERE site [19] and to the cAMP response element further upstream of

the RAP1 site [20–22]. Moreover, protection by Sp3 was observed at the RAP1-binding site and cAMP response element, and to a minor extent at the gERE site. Weak protection by Sp3 could be due to lower concentrations of recombinant Sp3 protein or the fact, that the Sp3 recombinant protein is not full-length in contrast to the Sp1 protein. The in vitro DNA–protein binding data correlated with the functional results. We here report Sp1- and Sp3-mediated activation of the gastrin promoter that was dependent on the functional RAP1 site.

Sp1 activation is a complex mechanism involving not only protein–DNA interactions, but also interaction of multiple modular domains of Sp1 with coactivator proteins [23,24]. Artificial and cellular promoters are differentially responsive to Sp3. Sp3 is believed to repress transcriptional activation of viral promoters, most likely by competing with Sp1 for the binding site [11,12,25]. Functional analysis using endogenous cellular promoters revealed Sp3-mediated repression of Sp1 activation dependent upon the promoter context and/or number of functional Sp1-binding sites. Thus, single GC boxes within the histone H4 or TK promoters which confer transcriptional activation via Sp1 binding were not responsive to repression by Sp3, while Sp1-mediated activation of the DHFR promoter, containing multiple functional G/C boxes was strongly repressed by Sp3 [26]. Sp3 also activates the histone H4 promoter significantly [26]. Sp3 may also activate some promoter fragments containing an RCE (retinoblastoma control element) [27]. Artificial reporter constructs with multimerized elements omitting adjacent regulatory elements appear not suitable for promoter studies since activation and repression occur in a promoter context-dependent manner. We performed the functional studies with an intact gastrin promoter construct comprising 119 bp upstream the TATA box including a single RAP1 site. The presence of a single RAP1-binding site, instead of artificial multimerized binding elements, could explain the non-responsiveness to the repressor function of Sp3. The activator and inhibitor function of Sp3 reside in different parts of the protein. Therefore, the transcriptional activity of Sp3 might be regulated in vivo by relief of inhibition [28], suggesting that the promoter context is important for Sp3 function. Binding analysis suggested that an additional protein is involved in RAP1-like binding activity, since a weak DNA–protein complex remained after the combined Sp1 and Sp3 supershift. This binding activity may correspond to the 70 kDa reported by Tillotson [5] since this protein appears to be a zinc finger protein with binding specificity different from Sp1 and Sp3. A complete definition of the role of Sp1 and Sp3 in gastrin transcription, however, clearly awaits a more detailed mutational analysis of this promoter. Many studies have shown that yeast and mammalian transcription factors share functional and structural features. Interestingly, besides the binding specificity, Sp3 and the yeast RAP1 protein can function both, as activators and repressors of transcription, are context-dependent regulatory proteins and contain discrete silencing and activation domains apart from their DNA-binding sites [1–3,6,11,12,28]. Protein–protein interactions most likely control their diverse regulatory functions. Taken together, the RAP1-like binding activity in islet cells corresponds to the mammalian transcription factors Sp1 and Sp3, that share common features with the yeast RAP1 protein.

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